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# Heterologous production and secretion of *Clostridium perfringens* $\beta$ -toxoid in closely related Gram-positive hosts

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## Abstract

The spore forming bacterium *Clostridium perfringens* is a widely occurring pathogen. Vaccines against *C. perfringens* type B and C are currently manufactured using  $\beta$ -toxin secreted by virulent *C. perfringens* strains. Large-scale production of vaccines from virulent strains requires stringent safety conditions and costly detoxification and control steps. Therefore, it would be beneficial to produce this toxin in a safe production host and in an immunogenic, but non-toxic form (toxoid). For high-level expression of  $\beta$ -toxoid, we cloned the highly active ribosomal *rpsF* promoter of *Bacillus subtilis* in a broad host range multicopy plasmid. In *B. subtilis*, we obtained high intracellular production, up to 200  $\mu\text{g ml}^{-1}$  culture. However, the  $\beta$ -toxoid was poorly secreted. The employed *rpsF* expression system allowed using the same expression plasmids in other heterologous hosts such as *Lactococcus lactis* and *Streptococcus pneumoniae*. In these organisms secretion of  $\beta$ -toxoid was ten times higher compared to the best producing *B. subtilis* strain. These results show the usefulness of the *rpsF* based broad host range expression system. © 2006 Elsevier B.V. All rights reserved.

**Keywords:** *Clostridium perfringens*  $\beta$ -toxin; *Bacillus subtilis*; *Lactococcus lactis*; Heterologous protein expression; Protein secretion; *rpsF* promoter; Vaccine production

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## 1. Introduction

*Clostridium perfringens* is the main cause of clostridial enteric disease in domesticated animals. *C. perfringens* types B and C secrete  $\beta$ -toxin that causes necrotic enteritis in cattle, sheep, pigs and goats (Songer, 1996; Springer and Selbitz, 1999). Vaccines against *C. perfringens* type B and C are currently manufactured using  $\beta$ -toxin produced by the virulent *C. perfringens* strain itself. The toxin is secreted into the

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medium, and the culture medium is harvested and used for subsequent vaccine development. However, the production of vaccines from a virulent strain requires time consuming detoxification and control steps and stringent safety conditions. Therefore, it would be beneficial to produce this toxin in a safe production host, preferably as a non-toxic variant of the protein, which still retains full immunogenicity. For high-level expression and production it will be necessary to use a strong promoter, preferably non-artificially induced by costly inducers. Potential useful promoter candidates are promoters of highly expressed housekeeping genes.

The gene coding for S6 ribosomal protein (*rpsF*) is such a gene. Recently, we have described the regulation of *rpsF* in *Bacillus subtilis*. In this bacterium *rpsF* forms an operon with *ssb* and *rpsR*, encoding single stranded DNA binding protein and ribosomal protein S18, respectively. In *B. subtilis* the *rpsF* promoter is highly active during logarithmic growth (Lindner et al., 2004). Since the *rpsF* promoter controls genes essential for growth, which are strongly expressed in fast growing cells, it is likely that this promoter has a growth dependent control mechanism. We hypothesized that this particular feature can be utilized when producing heterologous proteins that are toxic when produced at high levels. In theory: when a cell produces a protein that causes cell growth to slow down, a cell growth dependent promoter will slow down the transcription of the protein cloned behind it, resulting in an equilibrium with optimal growth rate and protein production. Another advantage of the use of a promoter like *rpsF* is that, due to the conserved function of these genes, the promoter might be active in different bacterial species. This would enable a quick assessment of production hosts with a single expression construct.

*B. subtilis* is a promising heterologous host for  $\beta$ -toxoid production. It has an established record for protein production and secretion and general safety. *B. subtilis* does not contain endotoxins, has been granted a GRAS status by the FDA, and can secrete proteins up to 20 g l<sup>-1</sup> yields (Harwood, 1992; Schallmey et al., 2004). Furthermore, like *C. perfringens*, *B. subtilis* is a Gram-positive organism, which increases the chance of success for production of  $\beta$ -toxin. In this study we have tested the applicability of the *rpsF* promoter as a high expression promoter for  $\beta$ -toxin production in *B. subtilis*. For this purpose we used a variant of the  $\beta$ -toxin,  $\beta$ -toxoid, that contains three point mutations render-

ing it no longer toxic, but still immunogenic (Segers et al., 1999). In addition, we have examined the effect of secretion on total production of  $\beta$ -toxoid. Finally, we have tested whether the *rpsF* promoter of *B. subtilis* can advantageously be used for  $\beta$ -toxoid production in other Gram-positive heterologous hosts, such as *Lactococcus lactis* and *Streptococcus pneumoniae*.

## 2. Materials and methods

### 2.1. Bacterial strains, medium and growth conditions

Table 1 lists the bacterial strains and plasmids used in this study. *B. subtilis* strains were grown at 37 °C under vigorous agitation in TY (1% tryptone, 0.5% yeast extract, 1.0% NaCl) or minimal medium (Spizizen, 1958). *L. lactis* strains were grown at 30 °C in M17 broth with 0.5% glucose (GM17) (Terzaghi and Sandine, 1975). *S. pneumoniae* was grown at 37 °C in GM17 broth. For the selection of transformants, appropriate antibiotics were added to the growth media at the following concentrations: for *B. subtilis* erythromycin 2  $\mu$ g ml<sup>-1</sup>, chloramphenicol 5  $\mu$ g ml<sup>-1</sup>, spectinomycin 100  $\mu$ g ml<sup>-1</sup>, hygromycin 125  $\mu$ g ml<sup>-1</sup>; for *L. lactis* chloramphenicol 5  $\mu$ g ml<sup>-1</sup> and erythromycin 5  $\mu$ g ml<sup>-1</sup>; for *S. pneumoniae* erythromycin 0.25  $\mu$ g ml<sup>-1</sup>. A 1% starch was added to solid media to visualise amylase activity when appropriate.

### 2.2. Strain constructions and transformation

The cloning and transformation procedures were performed according to established techniques (Bron and Venema, 1972; Sambrook et al., 1989) and supplier's manuals. Restriction enzymes, DNA polymerases, deoxynucleotides, T4 DNA ligase, plasmid isolation kits and PCR-product purification kits were obtained from Roche Diagnostics (Mannheim, Germany), and used as specified by the supplier. For all PCR-reactions PWO polymerase was used unless specified differently. Nucleotide sequences of primers used for PCR are listed in Table 2.

The *rpsF* and *yukE* promoter fragments were amplified by PCR from *B. subtilis* 168 chromosomal DNA using primers rpsF-3 and rpsF-5, and pyukE4-fw and

Table 1  
Strains and plasmids used in this study

Strains	Genotype	Reference
<i>B. subtilis</i> 168	<i>trpC2</i>	Anagnostopoulos and Spizizen (1961)
<i>B. subtilis</i> WB700	<i>nprE aprE epr bpr mpr::ble nprB::bsr Δvpr</i>	Wu et al. (2002)
<i>B. subtilis</i> WB800	<i>nprE aprE epr bpr mpr::ble nprB::bsr Δvpr wprA::hyg</i>	Wu et al. (2002)
<i>B. subtilis</i> DN2	168 <i>amyE::PhtrA-bgaB</i>	Noone et al. (2000)
<i>L. lactis</i> MG1363	Plasmid-free strain	Gasson (1983)
<i>L. lactis</i> NZ9000	<i>pepN::NisRK</i>	Kuipers et al. (1998)
<i>S. pneumonia</i> D39	Serotype 2, virulent strain, NCTC 7466	Avery et al. (1944)
Plasmids	Characteristics and description	Reference
pTRKH2	EryR, high copy, theta replicating	O'Sullivan and Klaenhammer (1993)
pDH32M	CmR, AmyE-integration vector	Kraus et al. (1994)
pRBT	pTRKH2:: <i>PrpsF</i> - $\beta$ -toxoid-gntT	This study
pRST	pTRKH2:: <i>PrpsF</i> - $\beta$ -toxoid without SS-gntT	This study
pRAT	pTRKH2:: <i>PrpsF</i> - <i>amyQ</i> -gntT	This study
pRN1	pTRKH2::NdeI–NdeI	This study
pYBT	pTRKH2:: <i>PyukE</i> - $\beta$ -toxoid-gntT	This study
pYST	pTRKH2:: <i>PyukE</i> - $\beta$ -toxoid without SS-gntT	This study
pYA <sub>ss</sub> BT	pTRKH2:: <i>PyukE</i> - <i>amyQ</i> -signal sequence-mature $\beta$ -toxoid-gntT	This study
pIV60	pDH32M:: <i>PrpsF</i> - $\beta$ -toxoid-gntT	This study
pNZ8048	reflective vector containing nisin inducible promoter Pnis	de Ruyter et al. (1996)
pNZbttox	pNZ8048:: $\beta$ -toxoid	This study
pKTH10	Replicative plasmid containing AmyQ	Darmon et al. (2002)
pBtox1	his6- $\beta$ -toxoid-his6	Intervet Int., Boxmeer, NL

pyukE4-rv, respectively. *B. subtilis yuke* is a gene of unknown function that probably has not correctly been annotated in the *B. subtilis* R16.1 annotation as available on SubtiList (Moszer et al., 1995), since the most likely promoter and ribosome binding site sequences overlap with the annotated open reading frame. Furthermore, in all annotated homologs found, the annotated first 8 amino acids are absent. We therefore assumed that not the first ATG codon but the second ATG codon, starting at basepair 25, is the actual start codon, and we have cloned the promoter accordingly.

The  $\beta$ -toxoid gene was amplified by PCR using primers Btox-RN1-fw and 2Btoxoid2 and pBtox1 plasmid as a template. The  $\beta$ -toxoid gene without signal sequence ( $\beta$ -toxoid-SS) was amplified from the same template using primers Btoxoid-ss+ATG and 2Btoxoid2. *amyQ* was amplified using primers AmyQ2-fw and AmyQ-rv and pTKH10 plasmid as a template. The *gnt*-terminator structure was amplified using Extensor polymerase (ABgene, Epsom, United Kingdom), *B. subtilis* 168 chromosomal DNA, and primers Gntterm-3 and Gntterm-2.

After amplification, the promoter fragments were digested by NdeI. The  $\beta$ -toxoid,  $\beta$ -toxoid-SS, and *amyQ* PCR fragments were digested by NdeI and BamHI. The *gnt*-terminator (*gnt*T) was digested BamHI. All digested fragments were purified using the High pure PCR-purification kit, and ligated in a three point ligation using T4 DNA ligase. The complete expression cassettes were subsequently amplified by PCR directly from the ligation mixture. When this PCR did not yield a single fragment, the fragment of the right size was isolated from gel using a High Pure PCR Product Purification Kit.

Purified expression cassettes were digested with PstI and HpaI, and ligated into a SmaI/PstI digested pTRKH2 high copy vector.

The single copy integration plasmid pIV60 was constructed by ligation of the *PrpsF*- $\beta$ -toxoid-*gnt*T cassette in pDH32m. To construct a fusion of the mature  $\beta$ -toxoid gene to the AmyQ signal sequence, the AmyQ signal sequence was amplified using primers AmyQfw-2 and AmyQ-ss-rv. The DNA fragment encoding mature  $\beta$ -toxoid was amplified using primers Btox-mature-fw and 2Btoxoid-2. Both fragments were

Table 2  
Primers used in this study

Primer name	Description	Sequence (5'–3') <sup>a</sup>
<i>rpsF</i> -3	Forward primer to amplify the <i>rpsF</i> promoter region	GGAATTCCTGCAGGTGACTTTGAGCGGGGCTTC
<i>rpsF</i> -5	Reverse primer to amplify the <i>rpsF</i> promoter region with RBS and ATG	CGTACTTTCTCATATGTTTGCACC
Gnt-term-3	Forward primer for <i>gnt</i> -terminator	GCGGATCCTACGTAACCTAATTAACCTGTATTAAAAACACG
Gnt-term-2	Reverse primer for <i>gnt</i> -terminator	GCTCTAGAGTTAACCTTCTGTTGTTTGGGATAG
Btoxoid-RN1-fw	Forward primer for $\beta$ -toxoid	GCGTCTAGACATATGAAGAAAAATTTATTTTCATTAGTTATAG
Btoxoid-ss+ATG	Forward primer for $\beta$ -toxoid without signal sequence	GCTCTAGACACATATGAATGATATAGGTAAACTACTACTATAA
2B-toxoid2	Reverse primer for $\beta$ -toxoid	CGCGGATCCTTAAATAGCTGTTACTTTTGTGAG
Btoxoid-RN2-fw	Forward primer for $\beta$ -toxoid cloning in pNZ8048	CGTTGCCATGGAGAAAAATTTATTTTCATTAGTTATAG
Btoxoid-RN2-rv	Reverse primer for $\beta$ -toxoid cloning in pNZ8048	CGCTCTAGATTAAATAGCTGTTACTTTTGTGAG
Btox-mature-fw	Forward primer for mature $\beta$ -toxoid, enabling fusion to signal sequences	CGGTCTCAAATGATATAGGTAAACTACTACTATAA
pyukE4-fw	Forward primer for yukE promoter region	GCAACTGCAGCGGAGGCAAGACTAATTAAG
pyukE4-rv	Reverse primer for yukE promoter region with RBS and ATG	TAATCCTGCCATATGCCTCATTACCT
amyQfw-2	Forward primer to amplify amyQ	CAATACGCATATGATTCAAAAACGAAAGC
AmyQrv	Reverse primer to amplify amyQ	GCGGATCCTCAGCTTGGAGGTGTTT
AmyQ-ss-rv	Reverse primer for AmyQ-signal sequence, enabling fusion with mature $\beta$ -toxoid	CGGTCTCACATTGGCTGATGTTTTTGTAAATCG
RNlacZ-fw	Universal primer on puc18 derived MCS	GTGAGCGGATAACAATTTACACACAGG
RNlacZ-rv	Universal primer on puc18 derived MCS	GGTTTCCCAGTCACGACGTTGTAA

<sup>a</sup> Underlined nucleotides indicate introduced hexameric restriction sites.

digested with Eco31I and ligated, resulting in a gene encoding an exact fusion of the AmyQ signal sequence to the mature  $\beta$ -toxoid at the signal peptidase cleavage site. The fused DNA was amplified by PCR from the ligation mixture and digested by NdeI and BamHI, and ligated in the likewise digested pYBT plasmid, resulting in pYA<sub>ss</sub>BT.

pNZbtox was constructed by ligation of a NcoI and PstI digested  $\beta$ -toxoid PCR product, resulting from a PCR on pBtox-1 using primers Btox-RN2-fw and Btox-RN2-rv, into the likewise digested vector pNZ8048. An overview of all constructed plasmids is given in Table 1.

All ligation mixtures were transferred to electro-competent *L. lactis* MG1363 or *L. lactis* NZ9000 using a Gene pulser (Biorad laboratories, Hercules, California), as described earlier (Leenhouts et al., 1989).

Colonies were selected on solid media for the appropriate antibiotic resistance and were subsequently screened by colony PCR. From positive clones plas-

mids were isolated, which were inspected by EcoRI-digestion and DNA-sequencing.

*B. subtilis* 168, WB700 or WB800 were transformed with the constructed replicative plasmids isolated from *L. lactis*, and selected on solid medium for erythromycin resistance. *B. subtilis* strain BIV60 was obtained by a double crossover of plasmid pIV60 into the chromosomal *amyE* gene of *B. subtilis* WB700. *S. pneumonia* D39 was transformed by growing the strain at 37 °C in GM17 to an OD<sub>600</sub> of 0.1; competence-stimulating protein CSP-1 (Pozzi et al., 1996) was used at 100 ng ml<sup>-1</sup> for inducing competence. Transformed cells were selected on solid medium for erythromycin resistance.

### 2.3. Protein isolation, gel electrophoresis and Western blotting

Strains were grown as described and growth was followed by taking OD<sub>600</sub> measurements every hour.

From an OD<sub>600</sub> of 0.7 onwards, every hour, 2.0 ml samples were taken from the cultures. The cells were immediately separated from the supernatant by centrifugation for 1 min at 14,000 rpm, and cells and supernatant were separately frozen in liquid nitrogen. When the sample collection was finished all samples were thawed on ice. Cell fractions of *B. subtilis* and *S. pneumoniae* were prepared for SDS-PAGE as described previously (Veening et al., 2004). For *L. lactis* this protocol was adjusted and the cells were protoplasted at 50 °C instead of the described 37 °C. Supernatant fractions were concentrated 10 times upon trichloroacetic acid (TCA) precipitation, and samples for SDS-PAGE were prepared as described previously (Laemmli, 1970). Proteins were separated by SDS-PAGE, and either stained with Coomassie Brilliant Blue directly, or transferred to a polyvinylidene-difluoride (PVDF) membrane (Molecular Probes Inc., Eugene, Oregon).  $\beta$ -Toxoid protein was visualised using a monoclonal anti- $\beta$ -toxoid antibody (Intervet Int., Boxmeer, NL), and a secondary horse radish peroxidase-conjugated goat anti-mouse antibody (Amersham Biosciences, Buckinghamshire, UK).  $\beta$ -Toxoid concentrations were estimated using dilution series loaded on a Coomassie brilliant blue stained polyacrylamide gel with a Bovine Serine Albumin standard as reference.

#### 2.4. $\beta$ -Galactosidase activity assay

To assay  $\beta$ -galactosidase activities, overnight cultures were diluted in fresh medium, and samples were taken at hourly intervals for optical density readings at 600 nm (OD<sub>600</sub>) and  $\beta$ -galactosidase activity determinations. The  $\beta$ -galactosidase assay, and the calculation of  $\beta$ -galactosidase units (Miller units), were performed as described (Miller, 1982), with the adaptations for the heat resistant bgaB variant as described by Darmon et al. (2002).

#### 2.5. Addition of $\beta$ -toxoid to growing cultures of *B. subtilis*

*B. subtilis* 168 was grown as described with a starting OD<sub>600</sub> of 0.01. OD<sub>600</sub> was measured every hour, and after 2 h of growth  $\beta$ -toxoid was added at final concentrations of 2.0 and 5.0  $\mu\text{g ml}^{-1}$ . Growth was followed by OD<sub>600</sub> measurements until cells were 4 h into stationary growth phase.  $\beta$ -Toxoid was obtained from a

*L. lactis* culture harbouring the pRBT plasmid. Whole supernatant of this *L. lactis* culture was concentrated using TCA precipitation. As a control, concentrated supernatant of a *L. lactis* strain containing an empty vector was used.

#### 2.6. Automated growth assays and nisin-induction of $\beta$ -toxoid production in *L. lactis* NZ9000

For automated growth assays a Genious microplate reader (Tecan Instruments, Maennedorf, Switzerland) was used, using 96 well flat bottom microplates containing 200  $\mu\text{l}$  of GM17 medium with antibiotics, inoculated with O/N grown *L. lactis* strains diluted to an OD<sub>595</sub> of 0.1. The strains were grown at 30 °C and the OD was measured every 20 min after orbital shaking for 15 s to prevent sedimentation. After 2 h of growth, nisin from a culture supernatant of strain NZ9700 (de Ruyter et al., 1996) was added to half of the samples at a 1:1000 dilution, and growth was monitored for another 10 h while measuring optical density. From a separate microplate, the cultures from five wells were pooled 2 h after nisin-induction. These samples were separated in cell and supernatant fraction, and analyzed for  $\beta$ -toxoid production and secretion.

#### 2.7. Biological activity of *L. lactis* produced $\beta$ -toxoid

Female mice weighing 18–22 g were immunized IP on day 0 and day 14 with 250  $\mu\text{l}$  antigen in Diluvac Forte as outlined in Table 3. The mice were bled on day 28.

The obtained sera were used in a competitive ELISA which was performed as follows. Monoclonal antibody against native  $\beta$ -toxin F126 was coated in 96 wells plate overnight. The plate was blocked for 2 h with 1% PBS-T-BSA. In a separate plate the mice sera were

Table 3  
Preparation of the vaccine

Vaccine	Approximately $\beta$ -toxoid concentration ( $\mu\text{g/ml}$ )	Mean titer $\pm$ S.D.
1	10.8	32 $\pm$ 15
2	5.4	14 $\pm$ 19
3	2.7	11 $\pm$ 13
4	1.3	15 $\pm$ 14
5	0.7	<4



titrated three-fold, native  $\beta$ -toxin was added as a fix concentration to all wells. The antibodies were allowed to react with the  $\beta$ -toxin for 30 min at 37 °C and then transferred to the Mab coated plate. The plates were incubated at room temperature for 1 h. Horse radish peroxidase conjugate rabbit polyclonal antibodies were added and incubated for 1 h. After washing the plates were colorimetrically developed with OPD. Titers were calculated as half of the maximum OD<sub>490</sub> of the negative control relative to the internal serum control, which was defined as titer 50 i.u. ml<sup>-1</sup>.

### 3. Results

#### 3.1. $\beta$ -Toxoid production in *B. subtilis*

Previously, we have investigated the expression pattern of the strong ribosomal promoter *rpsF* in *B. subtilis* (Lindner et al., 2004). To test suitability and efficacy of this promoter for heterologous expression of  $\beta$ -toxoid, we constructed the high copy theta replicating expression plasmid pRST, containing the *B. subtilis* *rpsF*-promoter and *C. perfringens*  $\beta$ -toxoid gene without its endogenous signal sequence. This construct was introduced in *B. subtilis* 168 and resulted in high intracellular  $\beta$ -toxoid expression, up to 200  $\mu$ g ml<sup>-1</sup> culture, corresponding to 25% of total intracellular protein (Fig. 1A).

To test a secretion construct of  $\beta$ -toxoid, a plasmid was constructed containing the *B. subtilis* *rpsF* promoter and the  $\beta$ -toxoid gene with its original clostridial signal sequence (pRBT). Unfortunately, this plasmid could not be stably maintained in *B. subtilis*. Several attempts to construct this clone failed or resulted in strains that grew poorly and lost the plasmid after overnight growth. A single copy integration into the ectopic *amyE* locus did yield a stable strain. However, hardly any  $\beta$ -toxoid could be detected in the medium (data not shown). To test whether this could be due to the expression vector used, we tested the production and secretion of AmyQ, the alpha-amylase of *B. amyloliquefaciens*, which is widely used for testing secretion efficiencies in *B. subtilis* (Vitikainen et al., 2001; Hyyrylainen et al., 2001; Westers et al., 2004). The introduction of the vector with the *rpsF* promoter in front of *amyQ* (pRAT) resulted in high-level secretion of alpha-amylase. This shows that the *rpsF* expression

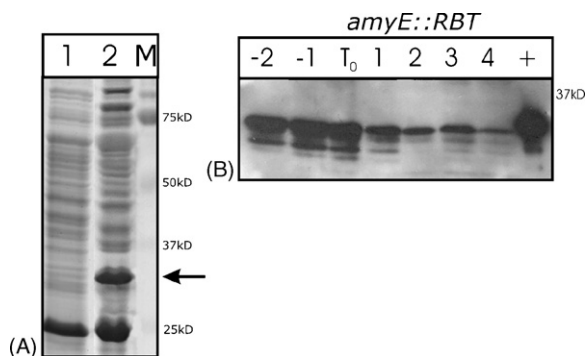


Fig. 1.  $\beta$ -Toxoid production in *B. subtilis*. (A) Coomassie brilliant blue stained SDS-poly acrylamide gel of whole cell extracts from *B. subtilis* 168 containing the pRST high copy vector or an empty vector control. Lane 1: 168 harbouring pTRKH2 (empty vector). Lane 2: 168 harbouring the pRST plasmid Lane 3 shows the protein marker (Dual stain, Biorad). The arrow indicates the  $\beta$ -toxoid protein. The abundant protein band at 25 kDa is likely to resemble the Erm protein conferring erythromycin resistance present on the pTRKH2 vector. The concentration of  $\beta$ -toxoid in lane 2 is 25% of the total extracellular protein. (B) Western blot of supernatant fractions of *B. subtilis* BIV60 culture containing a single copy integration of the expression cassette *PrpsF*- $\beta$ -toxoid integrated in *amyE*. Amounts loaded were corrected for OD. Numbers indicate time during growth in hours, where T0 is the transition from logarithmic growth to stationary growth. (+) Positive control (*C. perfringens*  $\beta$ -toxin).

system itself is capable of yielding high secretory levels.

To further investigate the bottlenecks in  $\beta$ -toxoid secretion we investigated the possibility that accumulation of secreted  $\beta$ -toxoid in the medium is toxic to *B. subtilis*. We therefore tested growth of *B. subtilis* in medium with externally added  $\beta$ -toxoid. No effect on growth was observed even when extracellular concentrations were five times higher than the maximal concentration observed in a  $\beta$ -toxoid producing strain; thus, low production is not a consequence of external toxicity (data not shown).

*B. subtilis* is well known for its capability of producing degradative enzymes, especially during stationary growth. It is therefore possible that  $\beta$ -toxoid is degraded when in it is secreted into the medium. To test this, the expression cassette was integrated in the *amyE* locus of a *B. subtilis* strain WB700 in which seven of the major extracellular proteases are deleted (Wu et al., 2002). In this strain, secreted  $\beta$ -toxoid was detected in the medium at concentrations of 0.1–0.5  $\mu$ g ml<sup>-1</sup> culture. Expression was highest

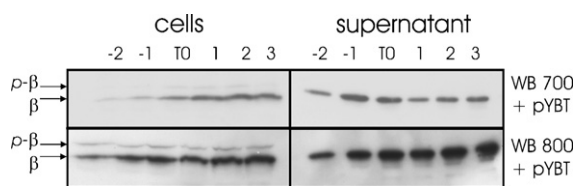


Fig. 2.  $\beta$ -Toxoid secretion in protease deficient *B. subtilis* mutants from the pYBT plasmid. Western blot showing cell and supernatant fractions of  $\beta$ -toxoid produced by two protease deficient *B. subtilis* strains, WB700 and WB800. Numbers indicate the time during growth in hours, where T0 is transition from logarithmic growth to stationary growth. Upper panels show intracellular accumulated and secreted  $\beta$ -toxoid in WB700, lower panels show intracellular accumulated and secreted  $\beta$ -toxoid in WB800. Amounts loaded were corrected for OD to be able to compare the production and secretion per cell. Supernatant fraction contain 4 $\times$  more culture volume compared to the cell pellets.  $\beta$ :  $\beta$ -Toxoid; p- $\beta$ : pre- $\beta$ -toxoid.

during logarithmic growth and still present in stationary growth (Fig. 1B).

Unfortunately, the use of a single copy integration of the expression cassette in the *amyE* locus results in the production of only low levels of  $\beta$ -toxoid. By using a different strong promoter it might be possible to obtain a stable multicopy construct carrying the  $\beta$ -toxoid gene. The *B. subtilis yuke* promoter is strong, however its expression timing differs from that of the *rpsF* promoter. Yuke expression is highest during the transition from logarithmic to stationary growth phase when the cells are grown in rich medium (MICADO, [http://locus.jouy.inra.fr/cgi-bin/genmic/madbase\\_home.pl](http://locus.jouy.inra.fr/cgi-bin/genmic/madbase_home.pl), Biaudet et al., 1997). Indeed, it proved to be possible to introduce the Pyuke containing vector into *B. subtilis* 168 and WB700 without severe effects on cell growth. As shown in Fig. 2, in *B. subtilis* WB700 the expression of  $\beta$ -toxoid started at the end of logarithmic growth and showed highest secretion around transition point. WB800 is the same as *B. subtilis* strain as WB700 except that the major cell-wall proteases *wprA* is deleted as well. Introduction of plasmid pYBT in this strain resulted in considerably more  $\beta$ -toxoid in the medium (0.5–1.0  $\mu\text{g ml}^{-1}$ ), showing the involvement of WprA in secreted  $\beta$ -toxoid breakdown. Notably, during all time points measured about twice as much  $\beta$ -toxoid was present intracellularly compared to the secreted fractions, indicating that secretion is a bottleneck in the production of  $\beta$ -toxoid in *B. subtilis* (Fig. 2). This was not caused by the signal

sequence since swapping with amyQ gave no improvement. To test whether the signal peptide of  $\beta$ -toxoid protein is the bottleneck we swapped it with the signal peptide of AmyQ, which can be secreted in *B. subtilis* to high amounts (Vitikainen et al., 2001). However, this did not yield more secreted  $\beta$ -toxoid, indicating that the signal peptide itself is not the limiting factor (data not shown).

### 3.2. $\beta$ -Toxoid secretion causes secretion stress in *B. subtilis*

To investigate whether the inefficient  $\beta$ -toxoid secretion may lead to a stress response in *B. subtilis*, we made use of strain DN2 (Noone et al., 2000) containing the promoter of *htrA* fused to a thermostable  $\beta$ -galactosidase gene integrated in the *amyE* locus (*amyE::P<sub>htrA</sub>-bgaB*). The *B. subtilis* HtrA protease is involved in the folding of secreted proteins and the degradation of malformed proteins after secretion (Pallen and Wren, 1997). Its expression is upregulated in response to overexpression of secreted proteins, the so called secretion stress response. This response is activated by the two component sensor system CssRS (Darmon et al., 2002). As shown in Fig. 3, the production of  $\beta$ -toxoid results in a strong induction of *htrA* expression. This effect is not observed when *cssS* is deleted or when  $\beta$ -toxoid was expressed without signal sequence (data not shown). Together these data clearly indicate that  $\beta$ -toxoid secretion results in secretion stress in *B. subtilis*.

### 3.3. The *rpsF* expression system in other Gram-positive bacteria

The *rpsF-ssb-rpsR* operon is conserved among many Gram-positive bacteria. An alignment of the upstream regions of the *rpsF-ssb-rpsR* operons of *B. subtilis*, *L. lactis* and *S. pneumoniae* was made using Genedoc (<http://www.psc.edu/biomed/genedoc>). As shown in Fig. 4, the promoter regions are highly similar. The pRBT plasmid (*P<sub>rpsF</sub>- $\beta$ -toxoid*) was introduced in *L. lactis* MG1363 and *S. pneumoniae* D39, and resulted in 5–10  $\mu\text{g ml}^{-1}$  of secreted  $\beta$ -toxoid (Fig. 5). In *L. lactis*  $\beta$ -toxoid up to 30% of the total secreted proteins in the medium consisted of  $\beta$ -toxoid. This considerable production shows that the *rpsF* promoter of *B. subtilis* is active in *L. lactis* and *S. pneumoniae*, and that



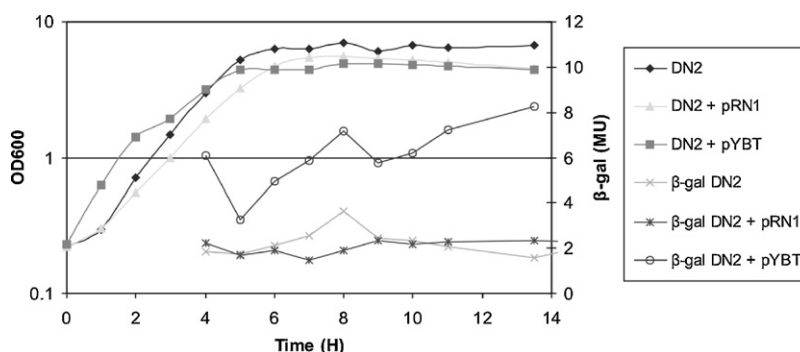


Fig. 3.  $\beta$ -Toxoid secretion causes specific secretion stress response in *B. subtilis*. Growth curves (closed symbols) and  $\beta$ -galactosidase activity (open symbols) of *PhtrA-bgaB* reporter strain containing either no plasmid (DN2), empty vector (DN2 + pRN1), or  $\beta$ -toxoid expression construct (DN2 + pYBT).

the  $\beta$ -toxoid signal sequence functions as an efficient export signal in both organisms.

To study the strength of the *rpsF* expression system in *L. lactis*, we compared it to results obtained with the nisin inducible expression system (NICE). The NICE system is generally regarded as one of the best production systems in *L. lactis* where the maximally produced amount of protein is limited only by the organisms production bottlenecks for the specific protein (de Ruyter et al., 1996). As shown in Fig. 5, the *rpsF* expression system yields similar levels of secreted  $\beta$ -toxoid as induction with the nisin inducible promoter, demonstrating the *rpsF* expression system provides a very efficient broad host range production platform.

### 3.4. Immunity and biological activity of $\beta$ -toxoid

To determine biological activity and immunogenic properties the  $\beta$ -toxoid was not purified. Instead total medium fraction of the cultures was used, which has

a clear cost-advantage over purification, and has no adverse effects on the animals health (Norton et al., 1994). Material produced in *L. lactis* MG1363 was tested for both biological activity and immunogenic properties. Toxicity of the  $\beta$ -toxoid was tested in mice. The  $\beta$ -toxoid, specifically constructed to have a reduced toxicity, is indeed non-toxic at levels at which the wild-type toxin is lethal. When 2.5  $\mu$ g/mouse (average weight 20 g) was administered 5/5 mouse survived. The  $\beta$ -toxoid was toxic when administered at 10  $\mu$ g/mouse (0/2 survived). These results show a toxicity reduction of three orders of magnitude compared to the WT  $\beta$ -toxin, which has a LD50 of 0.4  $\mu$ g/kg in mouse (i.v.) (Gill, 1982).

To test immunogenic properties of  $\beta$ -toxoid vaccines were prepared as described in the materials and methods section with  $\beta$ -toxoid concentrations as outlined in Table 3. The mice were immunized according to a standard protocol and 14 days after second immunization, the animals were bled. Anti  $\beta$ -toxin antibodies

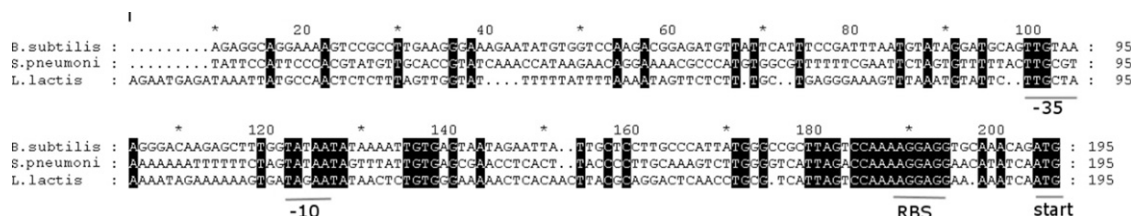


Fig. 4. Alignment of the *rpsF* promoter sequences. Alignment of *B. subtilis* 168, *L. lactis* MG1363 and *S. pneumoniae* D39 *rpsF* promoter regions. The start codon of *rpsF* (start), -35 and -10 promoter sequences, and RBS are indicated.

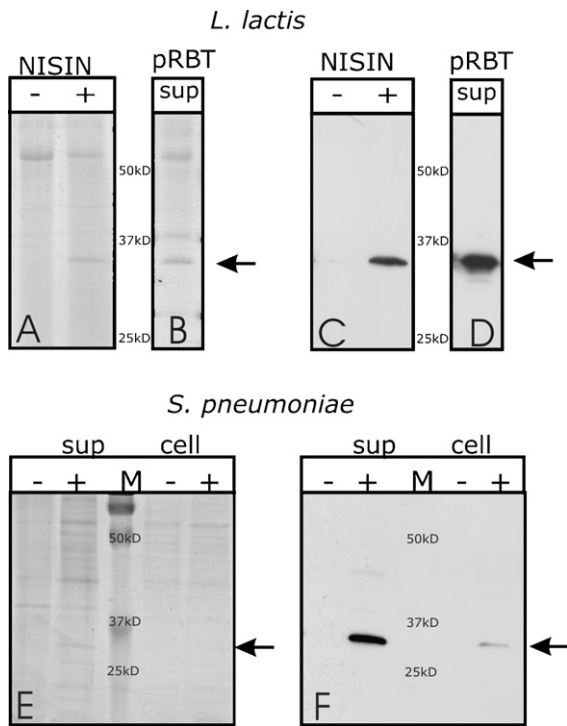


Fig. 5.  $\beta$ -Toxoid expression in *L. lactis* and *S. pneumoniae*. Upper panels:  $\beta$ -toxoid expression in *L. lactis*. (A) CBB stained PAA gel of culture supernatant of strain containing nisin inducible  $\beta$ -toxoid production plasmid, 2 h after addition of nisin (+) or buffer (–). (B) CBB stained PAA gel of culture supernatant of MG1363 containing the pRBT plasmid.  $\beta$ -Toxoid is indicated by the arrows. (C) Western blot of culture supernatant of strain containing nisin inducible  $\beta$ -toxoid, 2 h after addition of nisin (+) or buffer (–). (D) Western blot of culture supernatant of MG1363 containing the pRBT plasmid.  $\beta$ -Toxoid visualized using monoclonal anti- $\beta$ -toxoid antibody. Lower panels:  $\beta$ -toxoid expression in *S. pneumoniae*. (E) CBB stained PAA gel of culture supernatant and cell fractions, and (F) Western blot of culture supernatant and cell fractions,  $\beta$ -toxoid visualized using monoclonal anti- $\beta$ -toxoid antibody.  $\beta$ -Toxoid is indicated by the arrows. (–) Strain containing an empty vector control. (+) Strain containing the pRBT expression plasmid.

were determined as titers relative to WHO International Standard in a competitive ELISA which shows results comparable to European Pharmacopoeia (Ph.Eur.) (or 9 CFR test). Both Ph.Eur. and 9 CFR require that a vaccine induces 10 neutralizing international units in average in 10 rabbits, tested as neutralization in mice. All vaccine induced titers in mice with the exception of the vaccine preparation with lowest concentration of the antigen (Table 3).

#### 4. Discussion and conclusion

To obtain high yields of any heterologous protein, the use of a strong promoter is a prerequisite. In this research we show that the promoter of the *B. subtilis* ribosomal *rpsF* gene is a strong promoter that is capable of high expression of *C. perfringens*  $\beta$ -toxoid intracellularly. However, high production of  $\beta$ -toxoid in the growth medium turned out to be impossible. In fact, the presence of the complete  $\beta$ -toxoid gene (including secretion signals) under expression of the *rpsF* promoter on a high copy vector was lethal. Our evidence suggests that high expression of  $\beta$ -toxoid leads to a considerable stress, which, when pushed to the limits by a strong promoter as *rpsF*, is apparently toxic to the cells. These results also imply that the *rpsF* expression system does not autoregulate itself as we have postulated in Section 1. We anticipated to find that a cell growth dependent promoter would slow down the transcription of the protein cloned behind it when the produced protein causes stress that slows down the growth, resulting in an equilibrium with optimal growth rate and protein production. However, the stress on cell growth did not result in a sufficient decrease in  $\beta$ -toxoid expression to prevent plasmid instability and cell death.

*B. subtilis* is able to secrete several proteins up to  $20 \text{ g l}^{-1}$  (Schallmeyer et al., 2004), however it is known that secretion of heterologous proteins in *B. subtilis* is usually lower (Li et al., 2004). A frequently encountered problem with expression and secretion of heterologous proteins in *B. subtilis* is the rapid degradation of the secreted protein by extracellular proteases (Wu et al., 2002). It has also been reported that  $\beta$ -toxoid is sensitive to protease activity (Hunter et al., 1993). Therefore, it was not surprising to find that employing extracellular protease deficient *B. subtilis* strains increased the amounts of  $\beta$ -toxoid in the medium considerably.

##### 4.1. Secretion signals

It was previously shown, that adapting the signal sequence can improve protein secretion (Schallmeyer et al., 2004). The *C. perfringens*  $\beta$ -toxoid native signal sequence is a consensus Sec-type signal sequence, and in theory compatible with the *B. subtilis* secretion system (Tjalsma et al., 2000). Swapping the  $\beta$ -toxoid signal sequence with the signal sequence of a well

secreted *Bacillus* protein (AmyQ) did not increase its presence in the growth medium. Apparently, the  $\beta$ -toxoid signal sequence itself is not responsible for the poor secretion. These findings are in agreement with previous studies on the production of wildtype  $\beta$ -toxin, which has different properties than the  $\beta$ -toxoid, but contains the same signal sequence (Steinthorsdottir et al., 1998, 2000). Most likely the poor secretion and/or low extracellular stability is caused by characteristics of the mature  $\beta$ -toxoid protein, for instance by premature folding in the absence of suitable chaperones or rapid degradation due to slow folding kinetics directly after secretion at the cell membrane cell-wall interface. The expression of the gene encoding the *B. subtilis* HtrA protease is upregulated in response to overexpression of  $\beta$ -toxoid which is indicative for secretion stress (Fig. 3). Induction of HtrA is related to problems that occur in late stages of protein secretion (Leloup et al., 1997), presumably as a consequence of slow folding at the membrane-cell-wall interface (Braun et al., 1999). The response of the cells to secretion stress appears to be not sufficient to increase  $\beta$ -toxoid secretion, as the accumulation of non-secreted  $\beta$ -toxoid intracellularly indicates.

#### 4.2. Broad host range expression system

pTRKH2 is a stably replicating vector in a broad range of Gram-positive micro-organisms (O'Sullivan and Klaenhammer, 1993), making it a suitable broad host range vector. In addition, the *rpsF* promoter is well conserved in all Gram-positive organisms. Together, these genetic elements form a potential versatile protein expression system, which we have illustrated here by the heterologous production of  $\beta$ -toxoid in both *L. lactis* and *S. pneumoniae*. The expression yield of  $\beta$ -toxoid in *L. lactis* using the *rpsF* promoter was as high as the yield using the NICE expression system, showing that also in *L. lactis* the *rpsF* promoter on a high copy vector results in maximum obtainable expression levels of secreted protein in these organisms. As apparent from the results obtained, the secretion of  $\beta$ -toxoid in *B. subtilis* encounters bottlenecks that are less severe in *L. lactis* or *S. pneumoniae*. A likely bottleneck is degradation of the protein by a *B. subtilis* specific proteases that are absent in the other organisms. We are currently investigating the exact nature of this and other bottlenecks. With this study we have shown that an

opportunistic broad host range strategy can be advantageous to achieve optimal protein secretion yields, where it cannot, *a priori*, be predicted which host would be most suitable.

#### 4.3. Applicability

As shown by the immunity and biological activity experiments the produced  $\beta$ -toxoid has substantial reduced toxicity and still induces high titers in vaccination experiments. These results indicate that the  $\beta$ -toxoid is a very suitable vaccine component against *C. perfringens* type B and C.

$\beta$ -Toxoid in *L. lactis* was produced in culture flasks using standard medium. Induction of expression using the NICE system yielded sufficient amounts of  $\beta$ -toxoid for in small scale experiments. Until recently experience with large-scale application and fermentation of the NICE system was very limited. However, recent publications showed that the NICE system is well suited to produce target protein at industrial scale. Careful optimization of key parameters, like pH control, medium, and spore elements, leads to a significant increase in target protein yields (Mierau et al., 2005a,b), indication that also of  $\beta$ -toxoid commercially interesting yield are within reach.

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